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Among the signaling pathways regulated by growth promoting receptor kinases, one of the best characterized is the p42/p44 MAPK (Mitogen-Activated Protein Kinase) pathway. In this cytoplasmic kinase cascade, c-Raf Ser/Thr kinase phosphorylates and activates MEK1/2 (MAPK/ERK Kinase1 and 2) which in turn activate p42/p44 MAPKs by phosphorylation. MEKK (MEK Kinase), originally cloned based on sequence homology to yeast kinases BYR2 and STE11, also was able to phosphorylate and activate MEK1 *in vitro* and *in vivo* overexpression system. However, we found Raf and MEKK show different site preference in phosphorylating MEK, suggesting biochemically these two kinases are not identical. Unlike Raf, MEKK lacks the transformation capacity in cell transformation assay. Instead, expression of activated mutant of MEKK (DMEKK) displayed cell growth inhibition activity. *In vivo*, MEKK modulates a separate kinase cascade in which MEKK phosphorylates and activates SEK1 (also known as MKK4 or JNKK1) which is a direct upstream activating kinase of SAPK/JNK (Stress-Activated-Protein-Kinase/c-Jun N-terminal Kinase). In addition, MEKK fails to activate p38, another subfamily of MAPKs involved in stress response, and may even play a role in downmodulating the p38 pathway.

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Mutation and overexpression of growth promoting receptor kinases are often observed in different types of cancers, especially in breast cancer. As a result, this kind of disorder often leads to deregulation of downstream signaling cascades initiating from these receptor kinases. One of the best characterized signaling pathways is the p42/p44 MAPK (Mitogen-Activated Protein Kinase) pathway. In this cytoplasmic kinase cascade, p42/p44 MAPKs (also known as ERK1 and ERK2) are activated by upstream kinases MEK1/MEK2 (MAPK/ERK Kinase1 and 2) through phosphorylation of both Thr and Tyr residues organized in the TEY motif within kinase subdomain VII and VIII. The protooncogene product c-Raf Ser/Thr kinase is able to activate MEK1/2 by direct phosphorylation (Blenis, 1993). Another kinase MEKK (MEK Kinase) which was originally cloned based on sequence homology to yeast kinases BYR2 and STE11 also was found to be able to phosphorylate and activate MEK1 *in vitro* and *in vivo* overexpression system(Lange-Carter*et al.*, 1993). Our research interest will be focused on understanding the molecular mechanism of the signaling cascades involving Raf and MEKK.

Progress on specific aim 1

Our first specific aim is "To identify sites of phosphorylation on MEK1 by raf and MEKK1". This project has been finished and the result was published.(Yan and Templeton, 1994). Since this part has been included in last year's annual report, only the conclusions are listed here:

1. Both raf and MEKK are able to phosphorylate and activate MEK1 *in vitro* and *in vivo* overexpression systems.

2. Phosphorylation of Ser218 and Ser222 which lie in the activation segment of MEK1 is important for the activation of MEK1 both *in vivo* and *in vitro*. Substitution of Ser218 and Ser222 with negatively charged glutamic acid to mimic the phosphorylated serine residues renders MEK1 constitutively active.

3. Raf and MEKK1 differentially phosphorylate MEK1. Raf phosphorylates Ser218 and Ser222 equally while MEKK1 preferentially phosphorylates Ser218, suggesting biochemically raf and MEKK1 are different.

Progress on specific aim 2

We didn't pursue our second specific aim " To identify sites of phosphorylation on MEK and raf by MAPK" because other groups have

published the results before the approval of this fellowship(Brunet*et al.*, 1994; Gardner*et al.*, 1994; Mansour*et al.*, 1994).

Progress on specific aim 3

Because MEKK was originally identified as a kinase capable of phosphorylating and activating MEK1 *in vitro* and *in vivo* overexpression systems, we reasoned that MEKK might function as an oncogene as c-raf which was also able to phosphorylate and activate p42/p44 MAPK activator MEK1. c-raf consists of an N-terminal regulatory domain and a C-terminal kinase domain. Truncation of N-terminal regulatory region renders raf-1 constitutively active(Heidecker*et al.*, 1992). MEKK displays a similar structure: a putative N-terminal regulatory domain and a C-terminal kinase domain. C-raf has been clearly demonstrated as an oncogene and activated mutants of Raf are able to transform several types of cells including fibroblasts. Recently it was shown that active mutants of MEK1 were capable to transform fibroblast cells, suggesting MEK1 may function as a potential oncogene(Cowley*et al.*, 1994; Mansour*et al.*, 1994). Therefore our third specific aim will be focused on "To test MEKK activation following truncation of a regulatory domain, and to measure the ability of MEKK to function as an oncogene."

1. ΔMEKK, an N-terminal 367 amino acids deletion mutant of MEKK1 is active both *in vitro* and *in vivo*.
In an *in vitro* kinase assay using MEK1 as substrate, we found that ΔMEKK was highly active when it was purified from mammalian vaccinia overexpression system in CV1 cells or as a GST (Glutathione S-Transferase) fusion protein expressed in bacteria. In addition, ΔMEKK was also able to activate MEK1 *in vivo* in the vaccinia overexpression system.

2. Unlike active mutant of Raf and oncogenic mutant of ras , ΔMEKK fails to transform fibroblast cells.
In the cell transformation assay, we were not able to detect any transformation activity of ΔMEKK in NIH 3T3 cells and Rat-1 cells while oncogenic mutants of Raf and Ras exhibited strong cell transformation activities. This suggested MEKK might regulate different downstream signaling targets from those of Raf.

3. Overexpression of ΔMEKK is inhibitory to cell growth.
To investigate the function of MEKK *in vivo*, we have attempted to establish cell lines able to stably overexpress ΔMEKK using a CMV

promoter-based vector. However, we were unable to obtain stable cell lines that expressed detectable amount of ΔMEKK from NIH3T3 cells, Rat-1 cells, HeLa cells and CV1 cells. In parallel experiments, we successfully obtained cell lines that overexpressed MEK 2E (an active MEK mutant in which two glutamic acids substitute for the two serine residues phosphorylated during activation). Moreover, when ΔMEKK expression plasmid was cotransfected with plasmid carrying drug resistance marker, the number of colonies after drug selection was much fewer compared with that of the control transfection. Finally NIH 3T3 cells inducibly expressing ΔMEKK displayed a slower growth rate under induction condition (see below). All these suggested that stable overexpression of ΔMEKK was lethal or growth inhibitory to the cells which we have used.

4. Activation of SAPK/JNK pathway by MEKK.

To overcome the problem associated with constitutive overexpression of ΔMEKK, the LacSwitch system (Stratagene) was used to establish NIH3T3 cell lines inducibly expressing ΔMEKK. Several clones were isolated. Typically, the expression of ΔMEKK could be clearly detected after 12 hours of IPTG induction. One apparent consequence of IPTG induction was cell growth inhibition, a common characteristics of all inducible clones, even though to various extent. Actually, these cells demonstrated slower cell growth than parental NIH3T3 cells even without IPTG induction, possible due to leaky expression of ΔMEKK within these cells. The mechanism of this observed cell growth inhibition is yet to be determined.

Surprisingly, we found that in our IPTG inducible system where the expression level of ΔMEKK was moderate instead of being overexpressed, the p42/p44 MAPKs were not activated. Under the same condition, however, the SAPK/JNK (Stress-Activated-Protein Kinase/c-Jun N-terminal Kinase) subfamily of MAPKs was highly activated.

In contrast to p42/p44 MAPKs which are generally activated by mitogens such as growth factors and TPA (12-O-tetradecanoylphorbol-13-acetate), SAPK/JNKs are strongly activated by "stress" stimuli, including translational inhibitors (anisomycin and cycloheximide), UV irradiation, heat shock and proinflammatory cytokines (TNF-α and IL-1)(Cano and Mahadevan, 1995). Activation of SAPK/JNK requires dual phosphorylation on Thr and Tyr residues in a characteristic TPY motif

instead of the TEY motif of p42/p44 MAPKs(Derijard*et al.*, 1994; Kyriakis*et al.*, 1994). Similar to p42/p44 activators MEK1/MEK2, SEK1(also known as JNKK1/MKK4) is a dual specificity kinase capable of activating SAPK/JNK by phosphorylating both Thr and Tyr residues in the TEY motif of SAPK/JNK. SEK1 and MEK1/MEK2 are very specific towards SAPK/JNK and p42/p44 respectively(Sanchez*et al.*, 1994). We found that MEKK but not Raf-1 was able to activate SEK1 by phosphorylating both Ser and Thr residues organized in a similar region to those two Thr residues in MEK1/MEK2 phosphorylated by raf during activation. Therefore MEKK--SEK1--SAPK/JNK represents a distinct kinase cascade from the Raf--MEK1/MEK2--p42/p44 MAPKs cascade. These results have been published(Yan*et al.*, 1994).

5. p38 is not activated by MEKK.

p38 represents the third subfamily of mammalian MAPK. The defining sequence of p38 is the TGY motif which is also characteristic of yeast kinase HOG1 involved in osmotic response. p38, similar to SAPK/JNK, is preferentially activated by stress stimuli including protein synthesis inhibitors such as anisomycin and cycloheximide, LPS (lipopolysaccharide), proinflammatory cytokines (TNF-α and IL-1). Physical-chemical stresses such as UV irradiation, heat shock and arsenite also activate p38 strongly(Cano and Mahadevan, 1995).

Scince MEKK specifically activates SAPK/JNK but not p42/p44 MAPKs, it is interesting to know where MEKK is able to activate p38. Because activation of p38 is accompanied by increased tyrosine phosphorylation in the TGY motif(Han*et al.*, 1994; Raingeaud*et al.*, 1995), We examined the changes in tyrosine phosphorylation of p38 in NIH3T3 cells inducibly expressing ΔMEKK after IPTG induction. We found that under condition where SAPK/JNK was highly activated no increased tyrosine phosphorylation of endogenous p38 was detected. As control, both anisomycin (0.5 uM) and UV (100 J/m^2) treatments resulted in marked increase of p38 tyrosine phosphorylation (Fig.1).

It has been reported that SEK1 which is activated by MEKK is able to activate both SAPK/JNK and p38 *in vitro* and *in vivo* transient transfection experiments(Lin*et al.*, 1995). The failure of MEKK to activate p38 suggested that either SEK1 is not the physiological activator of p38 or the activation of p38 involves more complicated regulation rather than simple activation of the p38 activator SEK1. We are in the process of solving this puzzle.

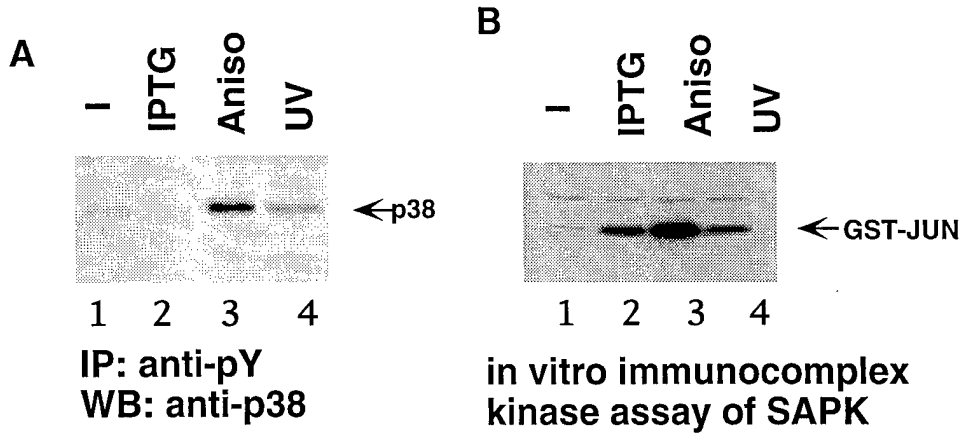

Figure 1. MEKK activates SAPK but not p38.
(A) NIH3T3 cells inducibly expressing ΔMEKK were untreated (lane 1), induced with 1 mM IPTG for 12 hr (lane 2), treated with 0.5 uM anisomycin for 30 min (lane 3) or exposed to UV (100 J/m^2) followed by incubation for 30 min at 37 ^{0}C (4). After cell lysis, half of cell lysates were subjected to immunoprecipitation by antiphosphotyrosine antibody pY20 (Transduction Laboratories). The immunocomplexes were then subjected to immunoblot against p38 (Santa Cruz). (B) Another half of cell lysates were used to measure the SAPK activities. SAPK was immunoprecipitated by anti-SAPK polyclonal antibody. In vitro immunocomplex kinase assay was performed using GST-Jun (amino acids 5-89) as substrate.

6. Inhibition of p38 activation by ΔMEKK.

To further investigate the role of MEKK in p38 activation, we examined the effect of ΔMEKK on activation of p38 by treatments known to stimulate p38. To do this, plasmid pEBG p38 expressing GST-p38 fusion protein was transfected into CV1 cells with or without pCMVΔMEKK. Expression of ΔMEKK completely blocked GST-p38 activation induced by anisomycin and $NaVO_3$ and partially by sorbitol (Fig.2). This suggested that MEKK downregulated the p38 pathway. The mechanism of MEKK induced downregulation is under investigation.

Recent studies have shown SAPK/JNK and p38 regulate different downstream targets. SAPK/JNK modulates transcription factor Elk, c-Jun and ATF2(Cano and Mahadevan, 1995). Activation of p38 results in increased phosphorylation of hsp27 (small heat shock protein p27) (Freshney*et al.*, 1994; Rouse*et al.*, 1994). It was also clear that p38 is important for the production of inflammartory cytokines such as IL-1, TNF-α, IL-6 and GM-CSG(Beyaert*et al.*, 1996). Therefore, it is possible that MEKK specifically activates SAPK/JNK while suppressing the activation of p38 to achieve specific biological consequence.

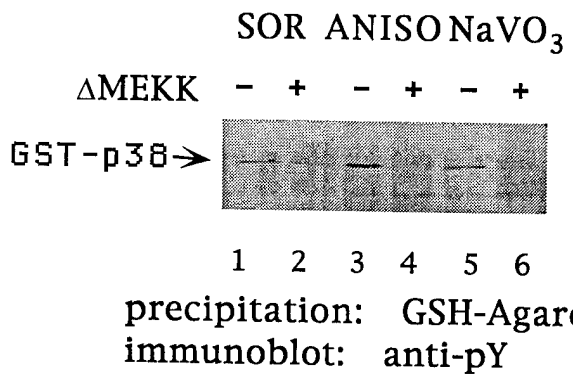

Figure 2. Inhibition of p38 activation by ΔMEKK.
pEBG p38 expressing GST-p38 fusion protein was cotransfected with pCMV-ΔMEKK (lane 2, 4 and 6) or with empty vector (lane 1, 3 and 5). Before cell lysis, cells were treated with 0.4 M sorbitol (lanes 1 and 2) , 0.5 uM anisomycin (lanes 3 and 4) or 2.5 mM $NaVO_3$ (lanes 5 and 6). GST-p38 was recovered by GSH-Agrose beads and subjected to anti-pY immunoblot.

CONCLUSIONS

Raf and MEKK show different site preference in phosphorylating MEK, suggesting biochemically these two kinases are not identical. Unlike Raf, MEKK lacks the transformation capacity during fibroblast transformation assay. Instead, expression of activated mutant of MEKK (ΔMEKK) displayed cell growth inhibition activity. *In vivo*, MEKK modulates a separate kinase cascade, MEKK--SEK--SAPK/JNK which is distinct from the Raf--MEK--P42/P44 pathway. In addition, MEKK fails to activate p38, another subfamily of MAPK involved in stress response and may even play a role in downmodulating the p38 pathway.

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